Bioaccumulation Rates, Acute and Chronic Effects of New Dielectric Fluid Products on Fish

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Toxicity and Bioaccumulation of Trichlorobenzene to Flagfish and Rainbow Trout

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Bioaccumulation Rates, Acute and Chronic Effects of New Dielectric Fluid Products on Fish

I: Toxicity and Bioaccumulation of Trichlorobenzene to Flagfish and Rainbow Trout

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Trichlorobenzene (TCB)

Water Exposure: Flagfish

1. Acute Toxicity

Standard 96-hour flow-through toxicity tests were conducted according to the methods described by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). The flow-through apparatus employed has been previously described (Smith et al. 1977). Water temperatures were maintained at 25 ± 1°C. Two age groups of flagfish were tested: 1) one week; and 2) two months.

Nominal TCB concentrations conformed with a recommended concentration series (Sprague, 1973): 2.4, 3.2, 4.2, 5.6, and 7.5 mg/l(ppm). Mean measured TCB concentrations ranged from 1.24 to 3.74 mg/l. Grab samples of water (250 ml) were analyzed for TCB prior to introducing the fish and every 24 hours thereafter, throughout the duration of the test (i.e. N=5). When possible, the chemical analysis was performed on both duplicate test tanks at each TCB concentration. Duplicates were considered acceptable if the higher measured concentration divided by the lower measured concentration did not exceed 1.2. Using these criteria, duplicate data was combined and a mean concentration per test level was reported. Similarly, mortality data for duplicates was combined for each test level.

The concentration of acetone carrier at all test levels was 196 mg/l (250 μl/l). Both water and acetone controls (in duplicate) were run simultaneously with each toxicity test.

Control mortality was usually 0%. When control mortality did occur, it was always $\leq 5\%$.

Data were analyzed by computer, utilizing an LC_{50} program developed by Stephan (1977). The program subjects the concentration/mortality data to a series of statistical tests which are progressively more sophisticated: 1) a simple binomial test; 2) the moving average test; and, 3) the probit analysis (if the concentration gradient tested produces at least two partial kills). The probit analysis, of course, is the most powerful test and hence yields the best estimate of the LC_{50}.

Table 1 summarizes the measured TCB concentrations for three independent acute toxicity tests.

Table 1. Test tank concentrations of TCB in mg/l during acute toxicity testing.

nominal TCB concentration	mean (±S.D.) measured TCB concentration (N=5) test #1	test #2	test #3
2.4	1.75 ± 0.15	1.43 ± 0.05*	1.24 ± 0.03
3.2	2.05 ± 0.12	1.81 ± 0.36	1.53 ± 0.04
4.2	1.56 ± 0.49*	2.43 ± 0.41*	2.43 ± 0.15*
5.6	2.62 ± 0.32	2.88 ± 0.66	2.57 ± 0.10
7.5	3.60 ± 0.10	3.69 ± 1.06*	3.74 ± 0.42

* concentrations not used in LC_{50} calculations

Concentration/mortality data at the nominal test concentration of 4.2 mg/l was not used to calculate the LC_{50} values for any of the acute tests. Measured TCB concentrations at this test level were sporadic, and tended to overlap with adjacent test concentrations. Moreover, mortality data between duplicates was often inconsistent. For the same reasons, concentration/mortality data at the nominal TCB concentrations of 2.4 and 7.5 mg/l for test #2 was not used in calculating the LC_{50}.

Tests conducted with one week old flagfish were repeated twice to confirm the initial LC_{50} value. Table 2 summarizes the acute toxicity of TCB. All LC_{50} values reported were calculated by probit analysis.

Table 2. Acute toxicity of TCB. 96-hour LC_{50} values for two age groups of flagfish.*

test number	age of flagfish one week	two months
1	2.17 (1.98-2.39)	3.18 (2.88-3.51)
2	2.28 (2.03-2.56)	
3	1.82 (0.58-5.69)	

* numbers in brackets denote 95% confidence limits

The first and second tests yield the best estimates of the LC_{50} for one week old flagfish. The LC_{50} reported in the third test is less reliable because of the wider confidence interval. The confidence interval associated with the LC_{50} for two-month old fish suggests that this estimate of acute toxicity is reasonable. As expected, the flagfish in the younger age group were somewhat more sensitive to the dielectric.

Two additional age groups of flagfish were also tested for acute toxicity: (1) one day; and (2) six months. Only the binomial test could be applied to the concentration/mortality data. This statistic will yield an LC_{50}, but no confidence interval. Hence, the values generated are less reliable. Day-old fry and six-month old fish yielded LC_{50}'s of 2.42 and 2.23 mg/l, respectively.

2. Chronic Toxicity

Juvenile flagfish were randomly distributed among the duplicate spawning tanks. Each tank received fifteen fish. All fish were maintained in non-dosed, control water during a one month growth and maturity period. Diet consisted of frozen adult brine shrimp, fed daily. Excess food and debris were siphoned daily from the tanks.

Following maturation, the fish were selectively culled to a ratio of five females to two males per tank, and spawning substrates were introduced. Once synchronous spawning in all test tanks was achieved, TCB addition was initiated.

Nominal TCB concentrations were: 0.32, 0.56, 1.0, 1.8, and 3.2 mg/l. The concentration of acetone carrier was 196 mg/l. Controls (water and acetone) were included in the test. Stable TCB concentrations were reached within the initial 24-hour dosing period. Water samples (250 ml) were analyzed five days per week from alternate, duplicate tanks at each test level. This sampling procedure and analysis continued throughout the duration of the chronic test.

Spawning substrates were removed daily and examined for eggs. Clean substrates were immediately substituted. Eggs were separated from the substrate and counted. Unfertile eggs and "shells" were discarded. A maximum of 50 viable eggs from each spawning were placed in individual egg cups. Cups were attached to a rocker arm assembly and the eggs incubated in their respective progeny tanks. Excess control eggs (from water control) were incubated at TCB concentrations where spawning had essentially terminated. Two hundred eggs per test level were used in this "control transfer" experiment.

Differences in egg production, viability and hatching success at different TCB concentrations were tested for significance by a one-way analysis of variance (ANOVA). If a significant F-ratio was found, a multiple range test (Duncan, 1955) was applied to determine which TCB concentrations yielded significantly different results.

Table 3 presents the measured TCB concentrations maintained during exposure of spawning adults and eggs.

Table 3. Mean test tank concentrations (± S.D.) of TCB in mg/l during exposure of spawning adults and eggs.

nominal TCB concentration	measured TCB concentration* spawning tanks (adults)	 Progeny tanks (eggs)
0.32	0.15 ± 0.01	0.22 ± 0.01
0.56	0.29 ± 0.03	0.34 ± 0.02
1.0	0.48 ± 0.05	0.55 ± 0.05
1.8	0.81 ± 0.11	0.93 ± 0.20
3.2	1.50 ± 0.20	1.71 ± 0.10

*N=18

Good control of TCB levels was achieved within this nominal concentration gradient. Measured quantities were approximately 50% of the TCB added. Magnetic stirrers, used in conjunction with the spawning tanks only, resulted in smaller measured quantities in this tank system.

No adult mortality occurred during the 21-day spawning period. There was, however, a decrease in general activity and feeding intensity at 1.5 mg/l TCB.

Table 4 summarizes the reproductive data collected during exposure of spawning adults and eggs.

Table 4. Reproductive response of flagfish exposed to TCB for 21 days.

parameter measured	water control^1	acetone control^2	nominal TCB concentration in mg/l 0.32^3	0.56^4	1.0^5	1.8^6	3.2^7
measured TCB (mg/l) in spawning tanks	0	0	0.15	2.29	0.48	0.81	1.50
number of spawnings	38	39	41	27	15	3	3
total eggs spawned	2088	2725	2945	561	252	6	11
mean daily spawn	49.7±43.7	64.9±59.9	70.1±54.1	13.4±28.0	6.0±13.3	0.1±0.7	0.3±1.4
mean per cent viable eggs	99.5±1.2	98.6±2.4	99.0±1.7	99.1±3.4	99.1±3.4	100±0	96.3±6.4
measured TCB (mg/l) in progeny tanks	0	0	0.22	0.34	0.55	0.93	1.71
mean per cent hatch	96.0±2.8	98.0±2.3	99.5±1.0	97.0±3.8	96.3±3.7	*	*

* insufficient number of viable eggs produced to yield statistically sound hatchability data.

	ANOVA	Duncan range test
mean daily spawn:	F = 30.4	3 2 1 4 5 7 6
mean per cent viable eggs:	F = 1.1	6 1 5 4 3 2 7
mean per cent hatch:	F = 47.9	3 2 4 5 1

Note: Numbered means not underscored by the same line are significantly different at the 1% level. Data from duplicate banks has been grouped.

There was a significant decrease ($P<.01$) in egg production at 0.29 and 0.48 mg/l TCB and essentially a termination of spawning above 0.8 mg/l TCB.

Egg viability at all TCB concentrations was virtually unaffected (i.e., viability > 96% at all exposure levels). However, with so few eggs produced at 0.81 and 1.5 mg/l TCB, the viable egg data may not be representative of exposure to these concentrations.

Although the ANOVA and range test statistics indicate significant differences in hatchability, it is unlikely that these differences are relevant. Both control groups, as well as the three TCB concentrations tested, showed good hatchability (i.e., > 96%). Moreover, water controls were not significantly different from the highest TCB concentration tested (i.e., 0.55 mg/l). Control eggs transferred to 0.93 mg/l TCB also showed a hatchability > 96%. At 1.71 mg/l TCB, control egg transfers exhibited a slightly reduced hatchability (i.e., ≃ 93%).

In a second experiment, control eggs were exposed to a higher TCB concentration gradient (i.e., nominal concentrations of: 1.8, 3.2, 5.6 and 7.5 mg/l). This experiment was conducted to find the TCB concentration which would produce a real effect on egg hatchability and fry survival.

Two hundred control eggs were incubated at each TCB concentration. Eggs incubated at nominal concentrations of 1.8 and 3.2 mg/l TCB served as controls against previous "egg transfer" hatchability data. Again, hatching success was tested for significance by a one-way ANOVA, followed by a multiple range test if a significant F-ratio was found.

One hundred fry, derived from the hatchability test, were retained at nominal TCB concentrations of: 1.8, 3.2 and 5.6 mg/l to determine survival over a 10-day period. Fry were transferred from the progeny tanks to the spawning tanks after hatching. Newly-hatched control fry were included in the survival test. Brine shrimp nauplii, fed once daily, served as a diet. Data collected on fry survival was subjected to Chi-square analysis.

Table 5 summarizes the test tank concentrations of TCB during exposure of control eggs and fry in the second experiment.

Table 5. Test tank concentrations of TCB in mg/l during exposure of control eggs and fry.

nominal TCB concentration	mean (± S.D.) measured TCB concentration progeny tanks (eggs)	spawning tanks (fry)
1.8	1.00±0.10 (N=12)	0.91±0.13 (N=16)
3.2	2.05±0.10 (N=11)	1.41±0.16 (N=16)
5.6	2.32±0.31 (N=12)	2.81±0.09 (N=14)
7.5	2.72±0.32 (N=12)	*

* no measurements made.

Good control of TCB concentration in the progeny tanks was achieved up to 2 mg/l (measured). Above this concentration, repeatability of the measurements decreased, as indicated by the standard deviations. No problems were experienced in controlling concentrations in the spawning tanks where the fry survival test was conducted. Again, this may be due to the use of magnetic stirrers with the spawning tank system.

Table 6 summarizes the hatchability data collected on control eggs incubated under the higher TCB exposure regime.

Table 6. Hatchability of control egg transfers exposed to TCB

parameter measured	nominal TCB concentration in mg/l 1.8	3.2	5.6	7.5
measured TCB (mg/l)	1.00	2.05	2.32	2.72
mean per cent hatch	96.0±1.6^{1}	91.5±1.9^{2}	91.0±3.8^{3}	60.5±2.5^{4}

	ANOVA	Duncan range test
mean per cent hatch:	F=7934.9	$\underline{1}\ \underline{2\ \ 3}\ \underline{4}$

Note: Numbered means not underscored by the same line are significantly different at the 1% level.

Differences generated by the ANOVA and range test with this set of data are real (i.e., F=7935). One mg/l TCB exposure yielded the same hatching success (96%) as 0.93 mg/l TCB did in the initial control egg transfer experiment. Hence, TCB exposure up to 1 mg/l probably does not have any significant effect on egg hatchability. In the first control egg transfer experiment, hatching success was 93% at 1.71 mg/l TCB. In the second transfer experiment, hatchability dropped to 91% when the measured TCB concentration rose slightly to 2.05 mg/l. Hatching success is significantly lower (P<.01) above 2.7 mg/l TCB. It is probably significantly reduced in the concentration range, 1.7 to 2.3 mg/l TCB.

Newly-hatched fry were retained in egg cups in the progeny tanks for 24 hours prior to being transferred to the spawning tanks. Initial exposure, therefore, occurred at TCB concentrations summarized in Table 6.

No fry survived the initial 24-hour exposure period at 2.72 mg/l TCB. At this concentration, as well as at 2.32 mg/l, fry retained their embryonic curvature following hatching. This phenomena severely reduced their locomotor activity.

Table 7 summarizes fry survival during exposure to TCB for 10 days, following transfer to the spawning tank system.

Table 7. Flagfish fry survival during exposure to TCB for 10 days.

day of test	measured TCB concentration in mg/l*			
	control	0.91 (1.8)	1.41 (3.2)	2.81 (5.6)
1	100	100	100	100
2	100	100	99	94
3	100	100	97	90
4	100	100	97	87
5	99	99	97	83
6	98	99	97	83
7	98	99	96	72
8	96	98	77	24
9	96	98	77	24
10	93	98	59	1

*numbers in brackets denote nominal TCB concentration in mg/l.

Chi-square analysis of mortality at 10 days: 98 93 59 1

Note: Numbers not underscored by the same line are significantly different at the 1% level.

Fry survival over a 10-day period was significantly reduced (P<.01) above 1.4 mg/l TCB. Figure 1 graphically depicts these dramatic differences in survival.

3. Bioconcentration

Bioconcentration testing of TCB with flagfish was conducted according to the Proposed Standard Practice for Measuring Bioconcentration of Chemicals with Fishes (ASTM - Draft #8, 1978).

Juvenile flagfish (≈six months old) were randomly distributed among four (two duplicate) spawning tanks. Each tank received 48 fish. They were fed frozen adult brine shrimp, twice a day. Tanks were siphoned daily.

Two nominal TCB concentrations were employed: 1) 1 μg/l (ppb); and 2) 10 μg/l. The concentration of acetone carrier was 98 mg/l. Fish were initially exposed to the TCB for a 28-day uptake period. During this uptake phase of the test, six fish (three per duplicate tank) were sampled for TCB body burdens after 1, 3, 7, 14, 21 and 28 days exposure. Sacrificed fish were randomly selected and killed by overdosing with MS-222.

The remaining fish were transferred to a clean flow-through system for a 14-day depuration period. During this phase of the test, six fish (three per duplicate tank) were sampled for TCB body burdens after 1, 2, 3, 4, 5, 7, 10 and 14 days. Again, fish were randomly selected and killed by overdosing with MS-222.

During uptake, water samples (250 ml) were taken daily (except weekends and holidays) from all four test tanks and analyzed for TCB concentration. A total of 20 samples per tank were analyzed.

Table 8 summarizes the test tank concentrations of TCB during the 28-day uptake phase of the bioconcentration test.

Table 8. Test tank concentrations of TCB in μg/l during 28 days of exposure.

parameter measured	low exposure (1.0)* right tank	left tank	high exposure (10.0)* right tank	left tank
measured TCB concentration in μg/l ($\overline{x}$±S.D.)	1.122 ±0.685	1.943 ±0.819	9.452 ±1.652	9.718 ±1.857

* numbers in brackets denote nominal TCB concentration in μg/l.

Note: N=20

Only acetone carrier (no TCB) was added to the low exposure test tanks. Previous acute and chronic TCB testing left background levels of ≈ 1 μg/l in the right bank tank and ≈ 2 μg/l in the left bank tank. Thus, the TCB concentrations reported in Table 8 for the low exposure level are actually the mean TCB background levels that were present in the system. This accounts for the variation between duplicates at this exposure level.

The high exposure did receive 10 μg/l TCB. Duplicates for this exposure level agree well. Moreover, standard deviations can be regarded as "tight", considering the length of the dosing period and the small TCB concentration involved.

Data collected on water and fish tissue concentrations of TCB were analyzed by a computer program called Biofac. The program, developed by the Dow Chemical Company, has been documented by Blau and Agin (1978). The abstract from this document best describes the usefulness of the program:

> Biofac is a computer program for determining the rate constants characterizing the uptake and clearance of chemicals from aquatic organisms. It consists of a nonlinear parameter estimation routine which generates optimal estimates of the rate constants from a set of sequential time-concentration data. These rate constants are then used to calculate a bioconcentration factor, K_B, which is the ratio of the concentration of chemical in the organism to the concentration in water at steady state. The data are weighted by a normality perserving transformation to reflect any time or concentration related trends in variability. The output from the program describes any uncertainty in the parameters as well as the validity of the model.

Biofac utilizes estimates of three important parameters derived from the time-concentration data: 1) the uptake rate constant, K_1^o; 2) the clearance rate constant, K_2^o; and 3) the heteroscedasticity parameter, γ. The program then iteratively searches for better values until the optimal values, K_1^*, K_2^*, and γ^* are found. Utilizing the optimal values for these parameters, Biofac then calculates: 1) the bioconcentration factor, BCF; 2) the depuration half-concentration, T(½); and, 3) the time required to reach 90% of steady state, T to 90% S/S.

Table 9 summarizes the Biofac analysis of our TCB data.

Table 9. Biofac analysis of TCB bioconcentration in flagfish exposed to nominal concentrations of 1 and 10 µg/l for 28 days.

parameter measured	units of measurement	right tank (1.122 µg/l)	left tank (1.943 µg/l)	right tank (9.452 µg/l)	left tank (9.718 µg/l)
K_1^*	1/g/day	1170	1699	744	834
K_2^*	$days^{-1}$	0.02	1.82	1.05	1.09
T(½)	days	0.75	0.38	0.66	0.64
BCF	-	1270	936	734	768
T to 90% S/S	days	2.50	1.27	2.18	2.12
γ *	-	1.996	1.999	1.999	1.999

Table 9 indicates that better agreement between duplicates for these parameter estimates was achieved at the high exposure level. This trend is consistent with the water concentration data reported in Table 8.

The Biofac analysis was also conducted by combining all of the time-concentration data available and normalizing the parameter estimates on the basis of a water concentration equal to unity (i.e., 1 µg/l).

Table 10 presents this final analysis which provides parameter estimates which are comparable with other laboratory studies of TCB bioconcentration. Figure 2 is a graphic output of this analysis.

Table 10. Biofac analysis of TCB bioconcentration in flagfish exposed to 1 µg/l for 28 days.

parameter	units	mean value ± standard deviation
K_1^*	l/g/day	1110 ± 310
K_2^*	days^{-1}	1.21 ± 0.36
T (½)	days	0.58 ± 0.17
BCF	-	921 ± 379
T to 90% S/S	days	1.91 ± 0.58
γ^*	-	1.999 ± 3.417

In an interlaboratory comparison (involving six international laboratories) of TCB bioconcentration in fishes, reported BCF's ranged from 48 to 2300, with an average value of 1126 (Branson, personal communication). The BCF and standard deviation in Table 10 agrees remarkably well with the reported average value.

Tissue samples (i.e., fillets) from several species of fish taken from the Thames River (Ontario) were analyzed for TCB body burdens. Species included in this analysis were: redhorse sucker, carp, white sucker, hognose sucker and small mouth bass. No TCB was found in any of these samples. Indeed, not even traces of the parent compound or metabolites were evident.

Eight fish samples (yellow perch) from Rice Lake (Ontario) were analyzed at a later date for TCB body burdens. When individually analyzed, all eight samples appeared to be "clean" with respect to TCB. The individual extracts were then pooled to yield six samples. GC analysis of the pooled extracts showed traces of both TCB isomers, as well as unidentified metabolite(s), present in all six samples (Fig. 3). Even though we could neither qualify nor quantify the new compound(s) detected, we could identify them as metabolite(s) of TCB. During the depuration phase of the flagfish test, TCB body burdens dropped quickly. By the fourth day following transfer of the fish to clean water, none of the 1, 2, 3-isomer (minor component) was detectable and only a trace (i.e., <0.01 µg/g) of the 1, 2, 4-isomer (major component) was present. However, five new peaks (only trace amounts) appeared in the chromatogram. By pooling several of the flagfish extracts and concentrating them, we were able to produce a reasonably good chromatogram of these metabolites. The unidentified peak(s) from the Rice Lake chromatograms matched those on the chromatogram from the pooled flagfish extracts. The Dow Chemical Company, Midland, Michigan, is presently conducting additional analytical work on our pooled flagfish extracts in an attempt to identify these compounds.

Food Exposure: Rainbow Trout

1. Bioaccumulation

Studies were also conducted on the bioaccumulation dynamics of TCB fed to rainbow trout via diet. This work employed a relatively new technique developed at the National Research Council of Canada. The methodology involves the incorporation of a radioactive "chemical marker" in the diet, in addition to the chemical of interest (i.e. TCB). The chemical marker used was ^{203}Hg-labelled methyl mercury.

The rationale underlying the method has been discussed in detail by Gidney et al., (1978) and Roberts et al., (1977). In practice, a group of fish is given a single feeding (i.e. a pulse dose) of contaminated ration. A post-dose series of whole body gamma counts is then used to construct a curve of the assimilation and clearance of the marker chemical. The ^{203}Hg-curve (Fig. 4) is comprised of a fast-clearing fraction (A) followed by a slow-clearing portion (B). The initial fast-clearing fraction represents clearance of the ingested dose of ^{203}Hg not absorbed from the gut tract. Clearance time for this portion of the curve coincides with voidance time of the contaminated meal. The slow-clearing portion of the curve can be back-extrapolated to the Y-axis (C) and gives a value, which divided by the initial whole body count, is taken as a measure of net assimilation efficiency, A_m (C/E) of the marker chemical (i.e. that portion of the ingested dose actually sequestered in the tissues).

Once voidance time is established, subgroups of fish are killed sequentially and analyzed for chemical of interest (i.e. TCB) body burdens. For each kill time, t, the ratio of TCB to ^{203}Hg content in the fish ($C^t{}_{TCB}/C^t{}_{Hg}$) is calculated. These ratios can be used to estimate the initial ratio, C^{o}_{TCB}/C^{o}_{Hg} (D). The net assimilation efficiency of TCB (A_{TCB}), can then be obtained from the estimated initial ratio and the corresponding ratio in the contaminated ration (C^{R}_{TCB}/C^{R}_{Hg}). Clearance of TCB from individual fish is calculated from tissue concentration at time of sacrifice and the estimated quantity initially sequestered in the tissues at zero time.

Two experiments, almost identical in methodology, were conducted to investigate assimilation and clearance of TCB in trout. The first study employed a ration containing 50 µg/g (50 ppm) TCB, while the second study utilized a ration dosed with 500 µg/g TCB (500 ppm).

New England Nuclear supplied the ^{203}Hg-labelled methylmercuric chloride (≈680 µCi/ml; 0.923 mCi/mg Hg). The stock isotope was diluted with 100 ml of 0.01N $CaCO_3$ solution. A commercial trout food served as the carrier material (i.e. diet).

In the first experiment, a 3 ml aliquot of diluted isotope was added to 5 mg TCB dissolved in 50 ml of absolute ethyl alcohol. The solution was sucked into a rotary evaporator flask containing 100 g of food. The flask was rotated in a water bath (48 - 50^{o}) for approximately six hours. Several 1 g samples of food were then analyzed for uniformity of both ^{203}Hg-label and TCB content. The percentage standard deviation for uniformity of both materials in the food was $<3.5\%$. The concentration of ^{203}Hg was 10,850 CPM/g (1.35 µg/g). TCB concentration was 50 µg/g.

Forty-five rainbow trout ($\overline{x}$ weight ± S.D. = 17.2 ± 2.83 g) were evenly distributed into five, 80 l tanks supplied by flow-through apparatus and allowed to acclimate for one week. Water temperatures during testing were maintained at 15 ± 1^{o}C. Following acclimation, the fish were starved for five days and then offered the contaminated ration at a rate of 0.5 g of food/10 g of body weight. They were allowed to feed _ad libitum_ for one hour. All uneaten food was then removed.

One minute gamma counts were conducted on all fish one hour after feeding. We utilized a modified whole body counting technique (Ruohtula and Miettinen, 1975) and an Ortec scintillation counter for conducting these measurements. Fish were anesthetized (MS-222) for this procedure. Following the initial count, the trout were re-distributed into twelve, 80 l test tanks. Fish placed into the same tank had dissimilar gamma counts. The whole body counts were used to identify individual fish, thus eliminating the need for physical tagging and possible infection problems. The number of individuals per test tank varied from 2 to 5 fish.

A random subgroup of 10 trout was used to establish the ^{203}Hg curve. Whole body counts were conducted on these fish for 44 days. Two counts per day were performed until void time was established (approximately 63 hours post-dose). Counting frequency was then reduced to one count daily for 7 days, followed by one count weekly for 5 weeks. All counts were corrected for radioactive decay, expressed as a fraction of the initial concentration of ^{203}Hg in the whole body, and the mean ^{203}Hg concentration for each post-dose count calculated.

Additional subgroups of four trout were counted, killed (overdosed with anesthetic) and analyzed for TCB body burdens at 79, 102, 174, 238 and 389 hours post-dose.

The remaining trout in the experiment were carried as "extras" in the event of an accidental overdose with anesthetic or regurgitation of the contaminated meal.

A non-linear regression analysis performed on the marker chemical data yielded an assimilation efficiency value, A_m, for the ^{203}Hg of 0.7312 (i.e., the y-intercept of the regression curve). The slope of this curve defines the clearance rate constant, K_m, of the marker chemical (i.e., the fraction of ^{203}Hg cleared per day from the tissues). The clearance rate constant is used to obtain a correction factor, F, for loss of ^{203}Hg which cleared from the tissues prior to analysis.

The initial ratio, C^o_{TCB}/C^o_{Hg} is obtained by non-linear regression analysis performed on data collected at 79, 102 and 174 hours post-dose. Only traces of TCB were found at sacrifice times of 238 and 389 hours and hence could not be included in the regression analysis.

The net assimilation efficiency of TCB is calculated as follows:

$$A_{TCB} = \frac{(C^o_{TCB}/C^o_{Hg}) \times A_m}{C^R_{TCB}/C^R_{Hg}} = 0.6077$$

where (C^o_{TCB}/C^o_{Hg}) = 0.00383 (y-intercept of the C^t_{TCB}/C^t_{Hg} regression curve)

A_m = 0.7312 (y-intercept of the ^{203}Hg regression curve)

C^R_{TCB} = 50 µg/g (measured TCB in ration)

C^R_{Hg} = 10,850 CPM/g (measured ^{203}Hg in ration)

Hence, for each gram of ingested ration containing 50 µg TCB, 30.4 µg TCB ($A_{TCB} \times C^R_{TCB}$) or 61% is assimilated.

The amount of ration ingested on an individual fish basis is calculated from the formula of Gidney et al., (1978):

$$R = \frac{M^D \times F}{C^R_{Hg} \times A_m}$$

where: M^D = concentration of ^{203}Hg at the time of sacrifice

$F = e^{k_m \cdot t}$ = correction factor for any loss of ^{203}Hg cleared from tissues prior to sacrifice.

The ingested dose, D, of TCB in the ration is calculated as follows:

$$D = C^{R}_{TCB} \times R$$

By calculating the initial quantity of TCB sequestered in individual fish ($D \times A_{TCB}$), and knowing the fraction of TCB remaining in the tissues when sacrificed, one can readily determine the per cent TCB cleared at sacrifice.

Table 11 summarizes the pertinent data on assimilation and clearance in individual trout fed a single meal containing 50 µg/g TCB.

Table 11. Assimilation and clearance in rainbow trout fed a single meal containing 50 µg/g TCB.

post-dose time, "t" (hours)	fish size (g)	ration ingested (g)	dose (TCB) ingested (µg)	TCB assimilated (µg)	TCB measured at "t" hours (µg)	% TCB cleared (at "t" hours)
79	14	0.644	32.2	19.6	9.4	52
	13	0.312	15.6	9.5	1.4	85
	18	0.539	26.9	16.4	8.2	50
	16	0.905	45.3	27.5	4.6	83
102	17	0.576	28.8	17.5	2.1	88
	16	0.659	33.0	20.0	2.9	86
	18	0.788	39.4	23.9	3.6	85
	15	0.813	40.7	24.7	3.6	85
174	19	0.574	28.7	17.4	0.9	95
	16	0.655	32.8	19.9	1.7	92
	16	0.693	34.7	21.1	1.5	93
	24	1.263	63.2	38.4	1.2	97

The second experiment, which utilized a ration containing 500 μg/g TCB, differed from the initial experiment in two ways: 1) a 9 ml aliquot (instead of 3 ml) of diluted isotope was added to the food to compensate for loss of radioactivity due to natural radioactive decay; and, 2) fish were sacrificed for TCB body burdens at 52, 76, 100, 143 and 218 hours post-dose (as opposed to 79, 102, 174, 238 and 389 hours post-dose in the first experiment). Mean weight (± S.D.) of the trout in the second experiment was 12.9 ± 2.74 g.

Analysis of the contaminated ration used in the second experiment indicated the concentration of ^{203}Hg to be 26,875 CPM/g. TCB concentration was 500 μg/g. The percentage standard deviation for uniformity of both chemicals in the food was $<5\%$.

The subgroup of 10 trout used to establish the ^{203}Hg curve had a void time of approximately 54 hours. Non-linear regression analysis performed on the marker chemical data yielded an assimilation efficiency value, A_m, for the ^{203}Hg of 0.726.

The initial ratio, C^o_{TCB}/C^o_{Hg}, was obtained by non-linear regression analysis performed on data collected at 75,100 and 143 hours post-dose. TCB body burdens measured at 52 hours post-dose were found to be pre-voidance measurements and could not be used. Only trace quantities were measured at 218 hours post-dose and, again, could not be used in the analysis.

The net assimilation efficiency of TCB, A_{TCB}, was 0.2711 (C^o_{TCB}/C^o_{Hg} = 0.00695).

Hence, for each gram of ingested ration containing 500 μg/g TCB, 135.6 μg TCB ($A_{TCB} \times C^R_{TCB}$) or approximately 27% is assimilated.

Table 12 summarizes the pertinent data on assimilation and clearance in individual fish fed a single meal containing 500 μg/g TCB.

Table 12. Assimilation and clearance in rainbow trout fed a single meal containing 500 μg/g TCB.

post-dose time, "t" (hours)	fish size (g)	ration ingested (g)	dose (TCB) ingested (μg)	TCB assimilated (μg)	TCB measured at "t" hours (μg)	% TCB cleared (at "t" hours)
76	17	0.630	315.0	85.4	24.5	71
	14	0.420	210.0	56.9	13.9	76
	12	0.160	80.0	21.7	3.4	84
	15	0.478	239.0	64.8	30.0	54
100	9	0.121	60.5	16.4	1.1	93
	13	0.280	140.0	38.0	15.6	59
	15	0.402	201.0	54.5	9.3	83
	14	0.475	237.5	64.4	13.2	80
143	11	0.112	56.0	15.1	0.7	95
	10	0.152	76.0	20.6	0.7	97
	12	0.434	217.0	58.8	7.5	87
	18	0.695	347.5	94.2	17.6	81

Both experiments gave the same assimilation efficiency for the marker chemical (i.e. ≃73%). There was, however, a considerable difference in assimilation efficiency of TCB (i.e. 61% and 27% for the first and second experiments, respectively). Even though the TCB dosage level was 10 times higher in the second experiment, the assimilation efficiency fell below 50% of that obtained in the low dose experiment.

If this difference is real, there must be limiting factors governing the uptake of TCB from the gut tract. These limiting factors may be related to metabolic processing rates. Perhaps there is an upper limit to the amount of TCB that can be assimilated (and cleared) per unit of time.

The per cent TCB cleared on an individual fish basis was more variable in the second experiment. We know that the higher concentrations of methyl mercury and TCB in the second experiment had no affect on the assimilation efficiency of the marker chemical. However, these higher concentrations may have affected the assimilation efficiency of TCB. Hence, we have less confidence in the clearance data with the 500 ppm ration. In future experiments, methyl mercury concentration in the ration will be $\lesssim$ 1 μg/g. At this level, it is assumed that the marker chemical is "biologically inert" (Gidney *et al.*, 1978).

Chemistry

1. Water Analysis

Using a method from B.D.H. Ltd. (1947), we determined the amount of acetone carrier in the test tank water. It is a colorimetric method involving the production of dinitro-phenyl hydrazones from 2, 4-dinitro-phenylhydrazine in the presence of acetone (i.e. ketones). We found that the nominal acetone carrier concentration of 196 ppm yielded a measured test tank concentration of 157 ppm.

New column conditions were adopted for the GC measurement of TCB. These conditions were according to the method of Karasek and Stepanik (1972) for the GC analysis of chlorobenzenes. The following new conditions provided better separation of the components in the TCB mixture (see Report #2, March, 1978 for previous column conditions):

Instrument: Hewlett-Packard 5730 a

Column: 8% Bentone-34, 10% DC-200 on Gaschrom Q, 80-100 mesh

Detector: ECD

Detector Temperature: 300°C

Injector Temperature: 250°C

Carrier Gas: Argon/Methane, 5%

Carrier Gas Pressure: 60 psi

Carrier Gas Flow: 50 ml/min.

Retention Time of 1,2,4-TCB: 2.10 minutes

Retention Time of 1,2,3-TCB: 5.00 minutes

Use of magnetic stirrers in conjunction with the flagfish spawning tanks was initiated to ensure more uniform TCB concentration. A comparative test was done involving a stirred and a non-stirred test tank over 5 consecutive days. In all cases the non-stirred tank gave slightly higher recovery results for TCB, but also less consistent results. Although stirring causes a higher evaporation loss of TCB from the system, the consistency of the analytical measurements from day to day was clearly improved.

In preparation for the flagfish bioconcentration test, we began practicing water extractions at the 1, 5, 10, 100 and 1000 ppb level, and found poor consistency in day to day measurements below 1000 ppb. Therefore, we tested the glassware used in the extraction process and found low TCB contamination on all glassware except stock bottles. To alleviate the problem, we incinerated the TCB residues by heating all the contaminated glassware to 570°C in an annealing oven after each use. Extractions of low concentrations (i.e. ppb range) of TCB from water immediately became much more consistent once this cleaning procedure was adopted. The procedure also improved the consistency of results at higher TCB concentrations (i.e. ppm range) which were used in the flagfish chronic test.

2. Tissue Extraction

The Dow method for tissue extraction of Aroclor 1016 was slightly modified for TCB. Hexane was used to extract the tissue, rather than ether. Tests showed that extraction efficiency of TCB is the same for both solvents.

Fish are placed in 20% methanolic KOH at 50°C for 20 minutes and the resulting digest extracted with non-spectro hexane. The hexane extract (containing TCB and lipid material) was allowed to separate from the caustic solution for approximately 15 minutes. The upper emulsion layer (hexane) was separated and dried over $Na_2 SO_4$. The hexane was then evaporated to 2 ml, applied to a Florisil column, eluted with 0.5% ether in hexane, and subjected to GC analysis. It was determined that TCB is recovered from the Florisil clean-up column in the first 80-90 ml of eluant. Both trout and flagfish tissue samples were spiked with known quantities of TCB to determine the suitability of the modified Dow method. TCB recovery was found to be 100%.

Some testing was done to determine if concentrating samples over a hot water bath caused evaporation of TCB along with the solvent. For samples in the concentration range, 10-15 ng/μl, only 80% of the TCB could be recovered. However, for samples with a concentration of approximately 1 ng/μl (i.e. the concentration used for GC injection), TCB loss by evaporation was negligible.

We also tested for evaporation loss of TCB from the caustic digestion process at three different temperatures: 1) room temperature (≈22°C); 2) 50°C water bath; and, 3) 90°C water bath. Three, 30 ml lots of methanolic KOH solution were each dosed with 70 mg TCB in 1 ml of carrier (methanol) and left for one hour under the prescribed temperatures. Each lot was then extracted with three, 20 ml portions of hexane and analyzed by GC. Recovery at room temperature and 50°C was 100%. At 90°C, however, recovery was only 70%. We therefore chose to conduct the digestion process at 50°C. At this temperature, digestion was quicker and no loss of TCB occurred.

The same extraction techniques used for fish tissues were applied to the trout food. TCB recovery from dosed trout food pellets was 100% at TCB concentrations of both 50 ppm and 500 ppm. Multiple sample analyses at both TCB concentrations also showed that the dielectric was uniformly distributed on the trout pellets.

Two alternative methods to the digestion process for TCB extraction from tissues were tried: 1) blending; and, 2) ultrasonic homogenization.

An attempt was made to blend a fish with the extracting solvent. The procedure resulted in poor TCB recovery (i.e. approximately 40% of the expected recovery).

Use of an ultrasonic homogenizer to blend fish tissue with solvent yielded only a 70% TCB recovery, relative to the KOH digestion method.

3. Solubility of Technical TCB in Water

No solubility data could be found in the literature on either of the pure components of the TCB mixture or on the mixture itself. Therefore, a study of the solubility of TCB in water was initiated.

The first experiment involved the supersaturation of 1 liter of water with TCB and subsequent overnight magnetic stirring of the mixture in an Erlenmeyer flask. Carefully avoiding any visible TCB droplets, two, 400 ml samples were withdrawn and extracted with 50 ml of hexane. Both samples gave solubility figures of about 40 ppm, upon GC analysis. The same test was repeated, with stirring omitted. Analysis gave solubility figures of about 15 ppm.

We repeated the first test (i.e. with stirring) and got very high numbers of about 75 to 100 ppm on one 400 ml sample. Another 400 ml sample was taken from the same lot and centrifuged at 20,000 rpm for approximately 15 minutes. A sample of the supernatant water gave a TCB concentration of 5.3 ppm. Because centrifuging dramatically reduced the solubility figure, we concluded that TCB must be present as a dispersion of tiny droplets. The 5.3 ppm figure, however, still seemed too high because plating out of TCB on the walls and bottom of the fish test tanks occurred at concentrations above 2 ppm.

Ultracentrifugation was also used to generate solubility figures for TCB in water. Ninety ml of a supersaturated TCB in water mixture were evenly distributed into six, 15 ml ultracentrifuge tubes and spun at 30,000 rpm and 5^oC for 60 minutes. Two samples were extracted with hexane and gave solubility values of 1.6 and 1.2 ppm.

A method for solubility of PCB's in marine and fresh water (Zitko, 1970) was used to determine water solubility of TCB. The TCB (1.5 ml) was added to 50 ml of distilled water in a blender and homogenized for 15 minutes. The resulting (hot) mixture was poured into 15 ml ultracentrifuge tubes and refrigerated for 30 minutes. After cooling, two samples were centrifuged for 30 minutes at 30,000 rpm and 5^oC. Hexane extraction and GC analysis yielded two very different results: 8 ppm and 27 ppm. The remaining four samples were centrifuged at 20,000 rpm and 5^oC for three hours. Solubility figures for these samples were: 25.5, 25.9, 28.7, and 26.2. We concluded that such high solubility figures were probably due to the formation of micelles or some form of a virtually unbreakable emulsion.

Some less violent means of equilibration was necessary to prevent the formation of emulsions. One such method (McAulfiffe, 1967) involved mixing an excess of TCB in water on a magnetic stirrer for 24 hours at a speed such that the vortex reached only 25% of the distance to the bottom of the flask. Four samples were then taken and spun at 20^{o}C for 2 hours at 20,000 rpm. Samples were withdrawn from the tubes and extracted with hexane. Resulting solubilities were: 0.45, 0.47, 0.53 and 0.46 ppm.

An intermediately violent type of agitation was tried next, involving the shaking of supersaturated TCB and water solutions in separatory funnels at maximum speed on a mechanical shaker for 15 minutes. Three samples were taken, centrifuged at 20^{o}C for 2 hours at 2,000 rpm, extracted, and analyzed. Resulting solubilities were: 0.87, 1.39 and 0.89 ppm.

Table 13 summarizes the solubility values obtained for TCB using the described methods of equilibration in conjunction with centrifugation.

Table 13. Summary of solubility methods and resulting values.

method and equilibration time	water solubility of TCB (mg/l)			
1. blender (15 min.)	25.45	25.90	28.65	26.15
2. magnetic stirring (24 hrs.)	0.45	0.47	0.53	0.46
3. mechanical shaking (15 min.)	0.71	0.87	1.39	0.89

Other solubility methods have also been investigated. Most of these methods involve coating of a glass vessel with a thin film of a chemical in volatile solvent (e.g. Haque and Schmedding, 1975). The solvent is allowed to evaporate, leaving a thin coat of the chemical on the walls of the vessel. Water is then added and equilibrated with the chemical over varying periods of time (i.e. 10 days to 6 months).

A test was also conducted to determine the effect of acetone carrier concentration on the solubility of TCB in water. Two acetone concentrations were tested: 1) 100 ppm; and, 2) 600 ppm. The solubility test method used magnetic stirring and centrifugation. Both acetone concentrations gave solubility figures of about 10 ppm.

From our investigation of TCB solubility in water, we must conclude that solubility is highly dependent on the method of equilibration. We also conclude that there is an urgent need for a standard method for liquid-liquid solubility. The ASTM was contacted to find out if a standard method exists. According to the reply, no acceptable method has been established.

4. Octanol-Water Partition Co-efficient

Fisher 1-octanol was purified by extracting 500 ml once with 100 ml of 0.1N NaOH, and twice with the same volume of distilled water. It was then distilled once under reduced pressure.

Several separate trials with varying volumes of octanol and water were conducted. First, 10 mg of TCB were weighed out and made up to 10 ml with octanol in a 10 ml volumetric flask. This mixture was added to 40 ml of distilled water in a 60 ml separatory funnel, and gently shaken for 15 minutes. The material was centrifuged at 12,000 rpm for 30 minutes at 5°C. A 10 ml water sample was withdrawn and extracted with hexane. The experimentally-determined co-efficient for this trial was 5.00. This value is somewhat high. The theoretical calculation (i.e. Hansch method) of the octanal-water partition co-efficient for TCB is 4.23.

Three separate trials were then conducted, following the previously described methodology, but using 25 mg TCB in 25 ml of octanal and 100 ml of distilled water. The following three values were found: 4.29, 4.12 and 4.10.

The same experiment was repeated six more times, with 50 mg TCB in 50 ml of octanol and 200 ml of water. The following six co-efficients were found: 4.72, 4.87, 4.80, 5.09, 4.74 and 5.00.

From our results, the octanol-water partition co-efficient for the TCB mixture is about 4.7 or 4.8, which is slightly higher than the calculated value (i.e. 4.23). The method is highly dependent upon good experimental technique. Experimentally-determined values approach the calculated value as one gains more experience with the method.

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FIGURE 1. SURVIVAL OF FLAGFISH FRY EXPOSED TO TCB FOR 10 DAYS.

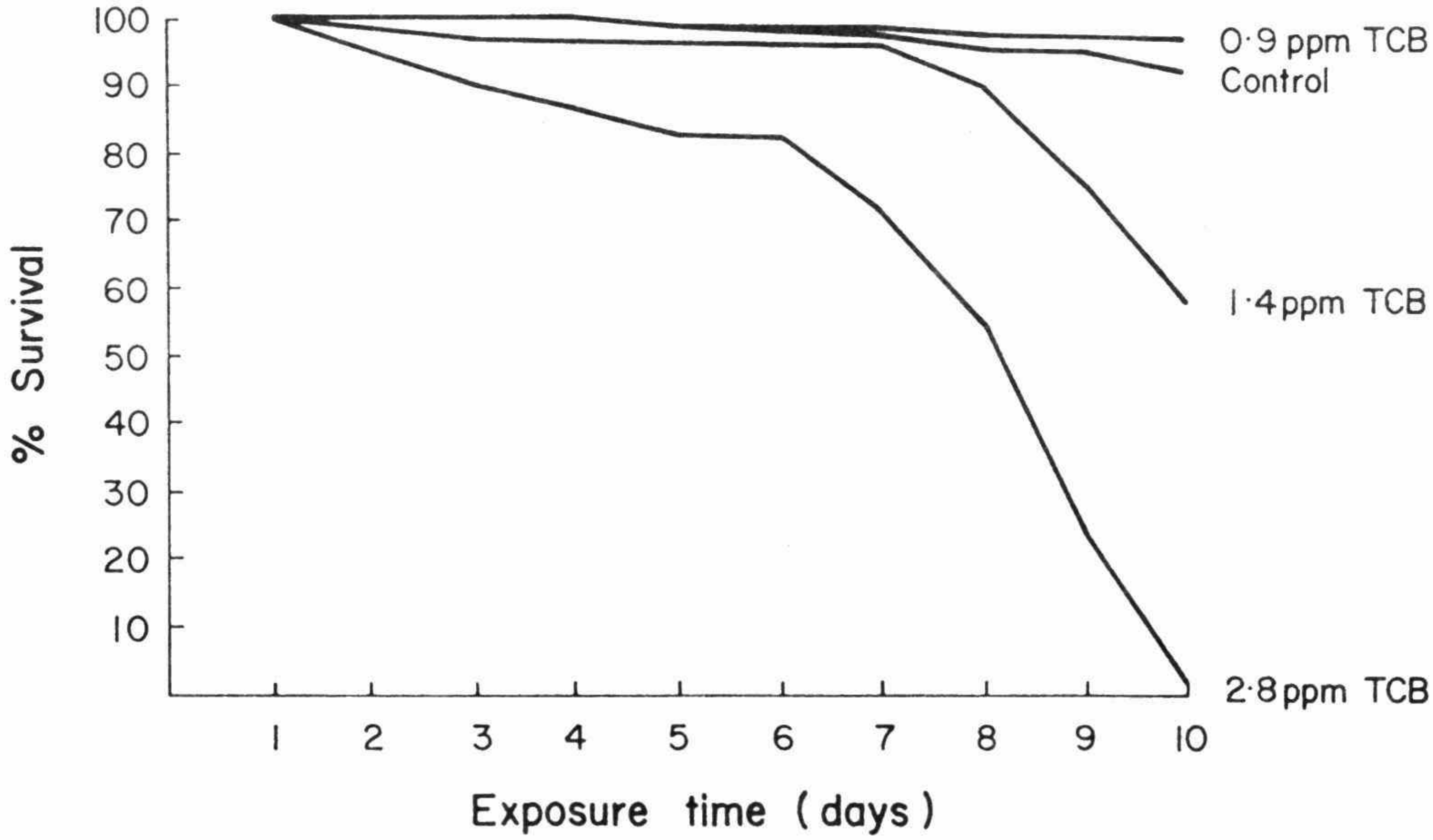
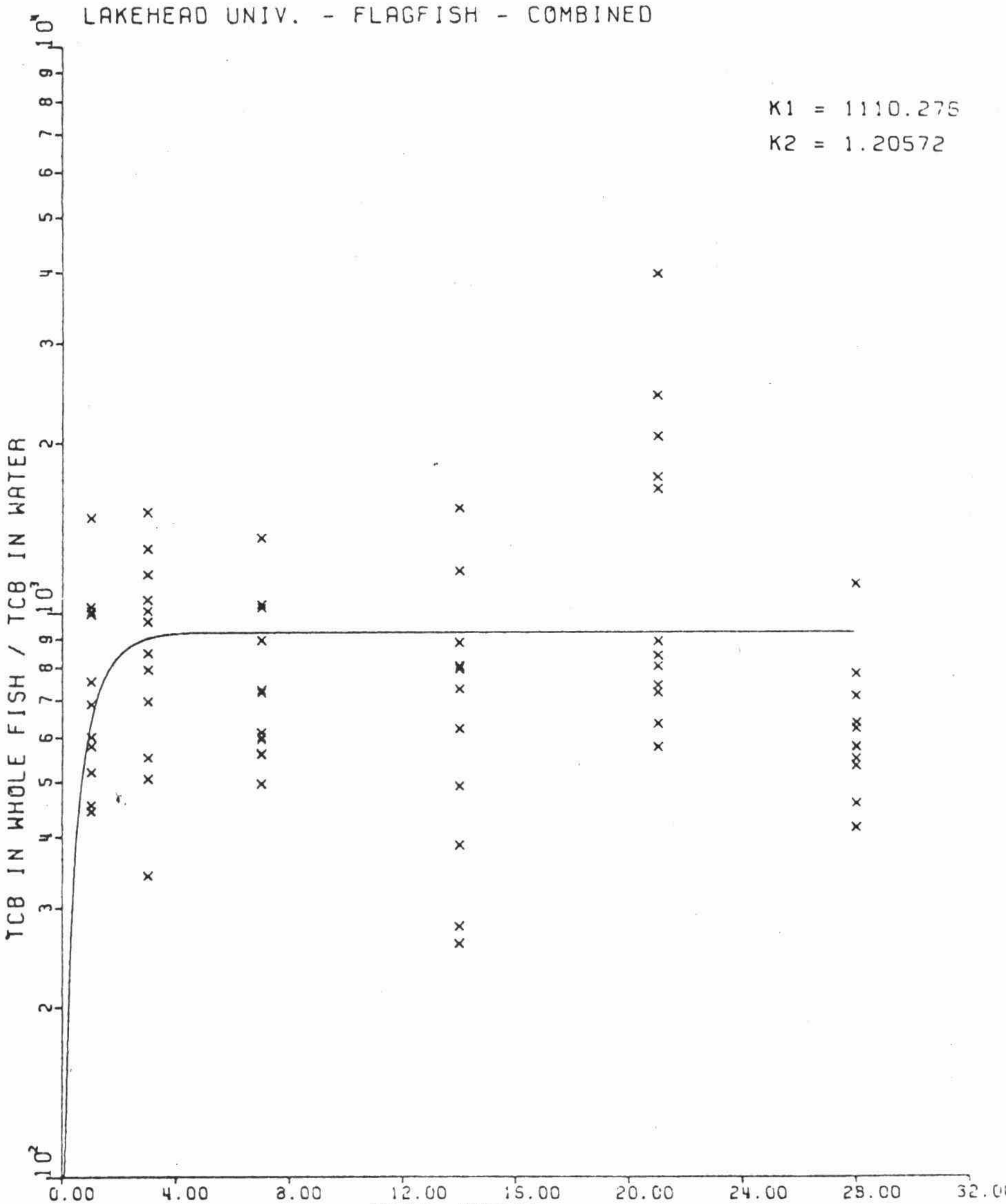


FIGURE 2. GRAPHIC OUTPUT OF BIOFAC ANALYSIS



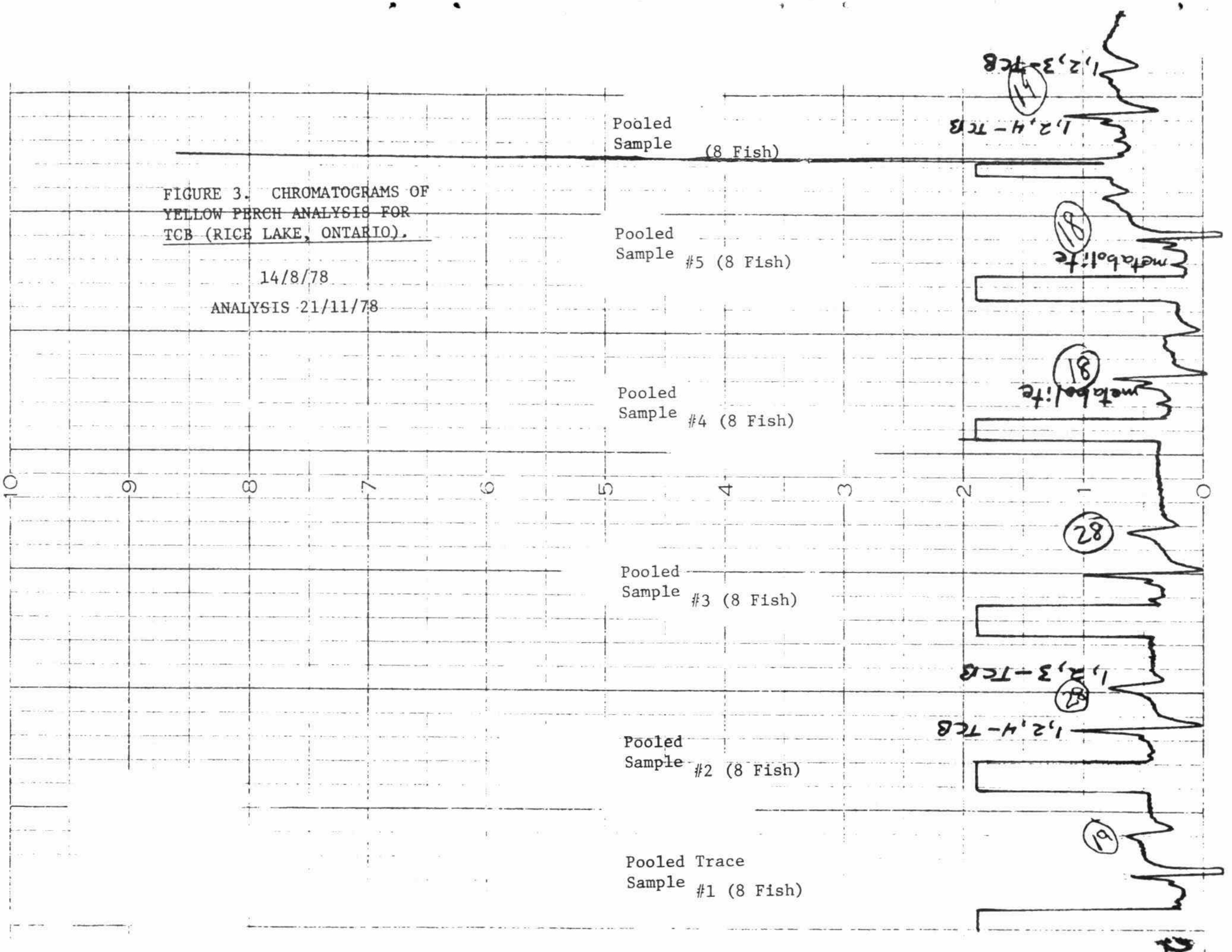


FIGURE 3. CHROMATOGRAMS OF YELLOW PERCH ANALYSIS FOR TCB (RICE LAKE, ONTARIO).

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ANALYSIS 21/11/78

Fig. 4. An illustration of two-compartment clearance of ^{203}Hg-labelled methyl mercury and its application in estimating the assimilation efficiency of any chemical of interest (modified from Gidney *et al.*, 1978).

The upper curve represents whole body retention of the marker (^{203}Hg) as a function of time. E is the initial count obtained after feeding of a single meal labelled with both marker and chemical of interest. Slope A represents clearance of the ingested dose of marker not absorbed from the gut tract. Slope B represents clearance of marker sequestered in tissues. C is obtained by extrapolation of slope B and is an estimate of the amount of marker sequestered in tissues at zero time. The ratio, C/E is an estimate of the net efficiency of assimilation for the marker, from the gut tract into body tissues.

The lower curve represents the ratio of amount of chemical of interest in the fish to the whole body count as a function of time. The ratios are calculated from direct measurements of marker and chemical of interest, determined on groups of fish sacrificed sequentially after voidance of the contaminated meal. D, obtained by extrapolation, is an estimate of the initial ratio. The net assimilation efficiency for the chemical of interest can be calculated from the initial ratio (D), the net assimilation efficiency of the marker (C/E), and the actual ratio of the chemical of interest to the marker in the ration.

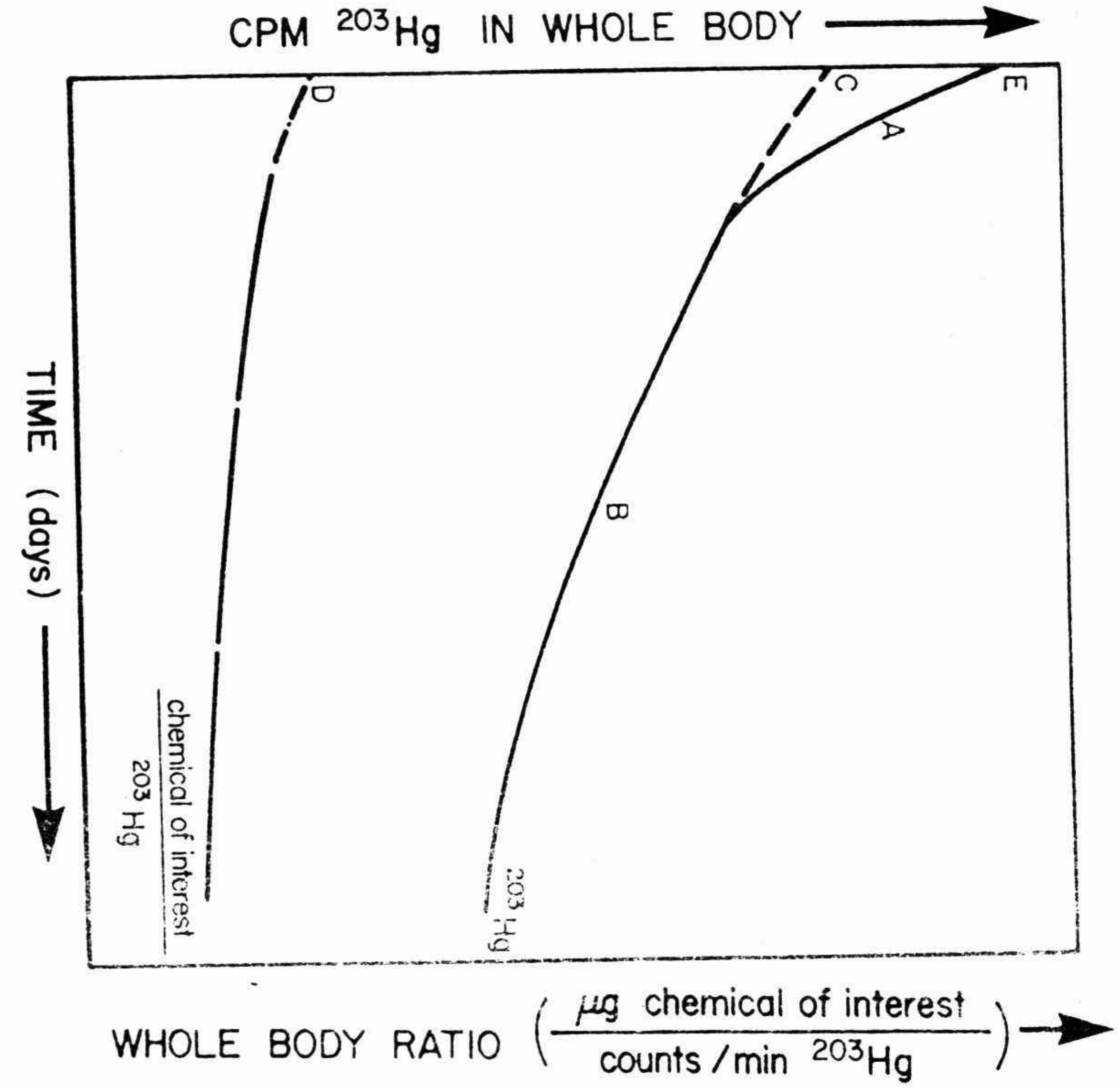
CPM 203Hg IN WHOLE BODY
E
C
A
D
B
TIME (days)
chemical of interest / 203 Hg
203 Hg
WHOLE BODY RATIO (µg chemical of interest / counts/min 203Hg)

FIGURE 4.

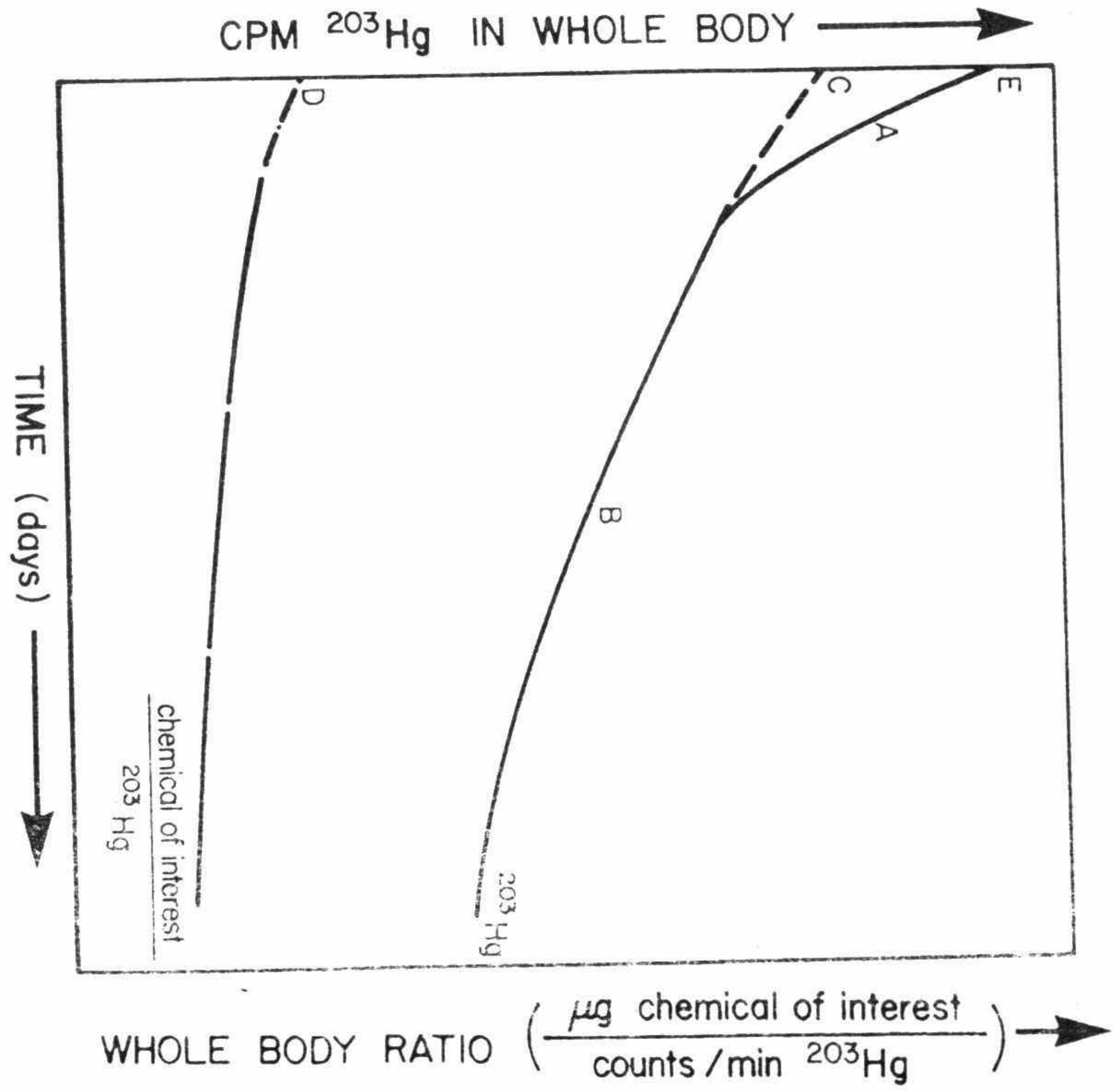
CPM ^{203}Hg IN WHOLE BODY
E
C
A
D
B
TIME (days)
chemical of interest / ^{203}Hg
^{203}Hg
WHOLE BODY RATIO (µg chemical of interest / counts/min ^{203}Hg)

FIGURE 4.